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1. E. J. of Human Genetics, Vol 6, pg 89-94, 1998

2. AMERICAN JOURNAL OF MEDICAL GENETICS, (1999 Dec 15) 88 (6) 694-9.

3. MOLECULAR PSYCHIATRY, (1999 Jan) 4 (1) 53-7. Journal code: CUM. ISSN: 1359-4184.

THANK YOU

Jeanine Enewold Goldberg
1655
CM1--12D11
306-5817

Article title	Polyglutamine-containing proteins in schizophrenia
Article identifier	1359418499000114
Authors	Joober_R Benkelfat_C Jannatipour_M Turecki_G Lal_S Mandel_J-L Bloom_D Lalonde_P Lopes-Cendes_I Fortin_D Rouleau_G
Journal title	Molecular Psychiatry
ISSN	1359-4184
Publisher	Macmillan
Year of publication	1999
Volume	4
Issue	1
Supplement	0
Page range	53-57
Number of pages	5
User name	Adonis
Cost centre	
PCC	$15.00
Date and time	Thursday, September 07, 2000 4:18:51 AM

18069302
Molecular Psychiatry (1999) 4, 53–57

ORIGINAL RESEARCH ARTICLE

Polyglutamine-containing proteins in schizophrenia

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Genetic anticipation, manifested by increased severity and earlier age-at-onset of the disease over successive generations, is reported in schizophrenia. The molecular basis of anticipation in several neurodegenerative diseases is unstable coding CAG repeat expansions. Anticipation was reported in schizophrenia. Recently, studies suggested that enlarged CAG/CTG repeats are over represented in schizophrenic patients compared to normal controls. Together, these observations suggest that unstable CAG repeats may play a role in the etiology of schizophrenia. The purpose of this study is to test for the presence of polyglutamine-expanded tracts, encoded by CAG repeats, in total protein extracts derived from lymphoblastoid cell lines of schizophrenic patients. Proteins from schizophrenic patients (n = 59) and normal controls (n = 73) were separated by means of SDS-polyacrylamide gel electrophoresis, wet blotted onto nitrocellulose membrane and probed with a monoclonal antibody (mab 1C2) recognizing expanded polyglutamine arrays. Three abnormal bands corresponding to protein(s) of molecular weight of approximately 50 kDa were identified in two unrelated schizophrenic patients and in a sibling of one of these patients. None of the normal controls tested positive for this abnormal band. These results suggest that expanded polyglutamine-containing proteins, though rare, may play a role in the pathogenesis of schizophrenia.

Keywords: schizophrenia; CAG repeats; polyglutamine tracts; anticipation; neuroleptic responsiveness

Introduction

Schizophrenia is a major psychiatric disorder that affects up to 1% of the general population. Family,[1] twin[2] and adoption[3] studies indicate that genes play a significant role in its etiology. However, no genes or loci increasing susceptibility to schizophrenia have been convincingly identified.[4] Genetic anticipation (ie earlier age-at-onset and increased severity of the disease in successive generations) has been reported in schizophrenia.[5–8] Genetic anticipation is observed in a group of neurodegenerative diseases caused by CAG repeat expansions. The severity of these diseases correlates with the size of the trinucleotide expansions, which are unstable during gametogenesis. It was therefore hypothesized that genetic anticipation observed in some families with schizophrenia may be due to unstable CAG repeat expansions.[9,10] Consistent with this hypothesis, several studies using the repeat expansion detection method reported a higher average maximum size of CAG/CTG in schizophrenic patients compared to normal controls.[11–13]

Although the exact role of expanded CAG repeats in the pathogenesis of neurodegenerative diseases is not clear, it is believed that their protein products are toxic to neural cells. Recently, it was shown that expanded polyglutamine-containing proteins tend to aggregate and form nuclear deposits which may represent one of the mechanisms leading to neural cell death.[14]

If CAG repeat expansions are involved in schizophrenia, they may also act through expanded polyglutamine tracts. To test this hypothesis, we used a monoclonal antibody (mab1C2) that specifically recognizes polyglutamine tracts to identify polyglutamine-containing proteins in total protein extracts derived from lymphoblastoid cell lines of schizophrenic patients and normal controls. The 1C2 antibody, initially raised against a peptide that encompasses the polyglutamine stretch of the TATA-binding protein (TBP),[15] was subsequently found to specifically recognize large (expanded[16] and non expanded[17]) polyglutamine arrays expressed in other proteins.

Methods

Subjects

The patients (n = 57) involved in this study are part of a currently ongoing pharmacogenetic project. In an attempt to reduce genetic heterogeneity, schizophrenic patients were categorized on the basis of *a priori* defined criteria of severity of the disease and respon-

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Received 20 May 1998; revised and accepted 26 August 1998

siveness to neuroleptic medication. Neuroleptic-nonresponsive patients (n = 28) were recruited according to the following criteria: (1) chronic schizophrenia, according to DSM-IV;[18] (2) continuous psychotic symptoms with no significant remission within the past 2 years; (3) at least three periods of treatment with typical neuroleptics, from at least two distinct families of drugs, at clinically sufficient dosage (equal to or greater than 750 mg, while treated in monotherapy, or 1000 mg chlorpromazine equivalent, while treated with two or more neuroleptics combined), for a continuous period of at least 6 weeks at a time, with no significant relief of symptoms in the preceding 5 years; and (4) unable to function without supervision in all or nearly all domains of social and vocational activities.

Criteria for neuroleptic-responsive patients (n = 29) were: (1) schizophrenia, according to DSM-IV; (2) at least one admission to a psychiatric care facility because of acute psychotic episode; (3) during all hospitalizations, full or partial remission in response to treatment with typical neuroleptics, at recommended dosage, within 6–8 weeks of continuous treatment (we define *remission* as a rapid reduction of schizophrenic symptoms with limited residual symptoms); (4) able to function with only occasional supervision in all or nearly all domains of social and vocational activities; (5) no admissions to hospitals because of psychotic exacerbation, if compliant to treatment and treated continuously with typical neuroleptics; and (6) at least one psychotic relapse when neuroleptic medication is reduced or interrupted. Exclusion criteria for schizophrenic patients were brain trauma, any neurological condition and drug or alcohol abuse in the last 2 years.

Neuroleptic-nonresponsive patients were selected from a list of schizophrenic patients identified as candidates for treatment or treated with atypical neuroleptics because of treatment resistance. Three institutions provided these nonresponsive patients: Douglas Hospital, Clinique Jeunes Adultes of LH Lafontaine Hospital and the Schizophrenia Clinic of the Royal Ottawa Hospital. Responsive patients were selected from a list of all patients who were considered very good responders to neuroleptics by their treating physicians and/or nurse and who were followed in the out-patient clinics attached to the Douglas and LH Lafontaine hospitals.

Patients were directly interviewed using the Diagnostic Interview for Genetic Studies[19] and their medical records were comprehensively reviewed by a research psychiatrist. Complementary information from the treating physician and nurses in charge was obtained. Diagnosis was based on the concordance of two psychiatrists using DSM criteria and all the available data. Diagnoses in relatives were based on direct interview using the DIGS and review of the medical files.

A group of 34 normal controls was recruited through local advertisement and among healthy married-in individuals from an ongoing linkage study. All controls have been screened for DSM-IV axis I disorders. In addition, 39 psychiatrically unscreened control subjects were tested. All patients and controls were Caucasians with western and central European descent. After complete description of the study to the subjects, written informed consent was obtained.

Protein extraction and western blotting

Whole-cell protein extracts were isolated from lymphoblastoid cell lines transformed by Epstein–Barr Virus using standard techniques. The protein extraction was performed on ice and the PMSF protease inhibitor was added (1 mM final concentration) to the protein homogenate. Fifty micrograms of proteins from each sample were separated using an 8% SDS-PAGE gel. Gels were blotted onto nitrocellulose transfer and immobilization membrane (Schleicher & Schuell, Keene, NH, USA) using BIO RAD Trans-Blot electrophoretic transfer cell apparatus following manufacturer recommendations (Biorad, Hercules, CA, USA). The quality of protein transfer and the amount of proteins were checked using the Ponseau red staining method. Blots were probed overnight at 4°C with the primary antibody (mab1C2; 1:5000), then detected using the horseradish peroxidase-conjugated secondary antibodies (1:4000) (Jackson Immino Research Laboratories, Baltimore, MD, USA) and enhanced chemiluminescence (ECL protocol, Amersham, Little Chalfont, Bucks, UK).

Results

Many immunoreactive bands were observed in all schizophrenic patients and healthy volunteers indicating that there are proteins, as yet uncharacterized, detected by the 1C2 antibody. The strongest signal, corresponding to the TATA-binding protein (with a size <48 kDa), was present in all subjects and may be considered as an internal positive control for each subject.

Two strongly immunoreactive bands were detected in two schizophrenic subjects (Figure 1, respectively lanes S1 and S4) but not in the 73 controls. Although both of them are of approximate molecular weight of 50 kDa, the band observed in S4 seemed to be slightly smaller than the one observed in S1. To ensure the reproducibility of these bands, proteins were extracted from at least two different cell line aliquots established from the two patients and western blots were repeated using different experimental stringency conditions. These bands were consistently identified under several experimental conditions. The immunoreactivity of the 50-kDa band detected in S1 was equivalent to that of the TBP band when the blot was incubated at room temperature for 1 h with the primary antibody at a 1:2000 concentration (Figure 2). In order to exclude the presence of immunoglobulins in these samples (S1 and S4) that would react with the secondary antibody, we repeated the immunodetection using only the secondary antibody. No abnormal bands were detected. Both patients were diagnosed with paranoid schizophrenia and were good responders to neuroleptic medication. S4 had no family history of mental disorders and his age-at-onset was 25 years (average of the group 21 ± 5 years). S1 had an earlier age-at-onset (19 years) and had

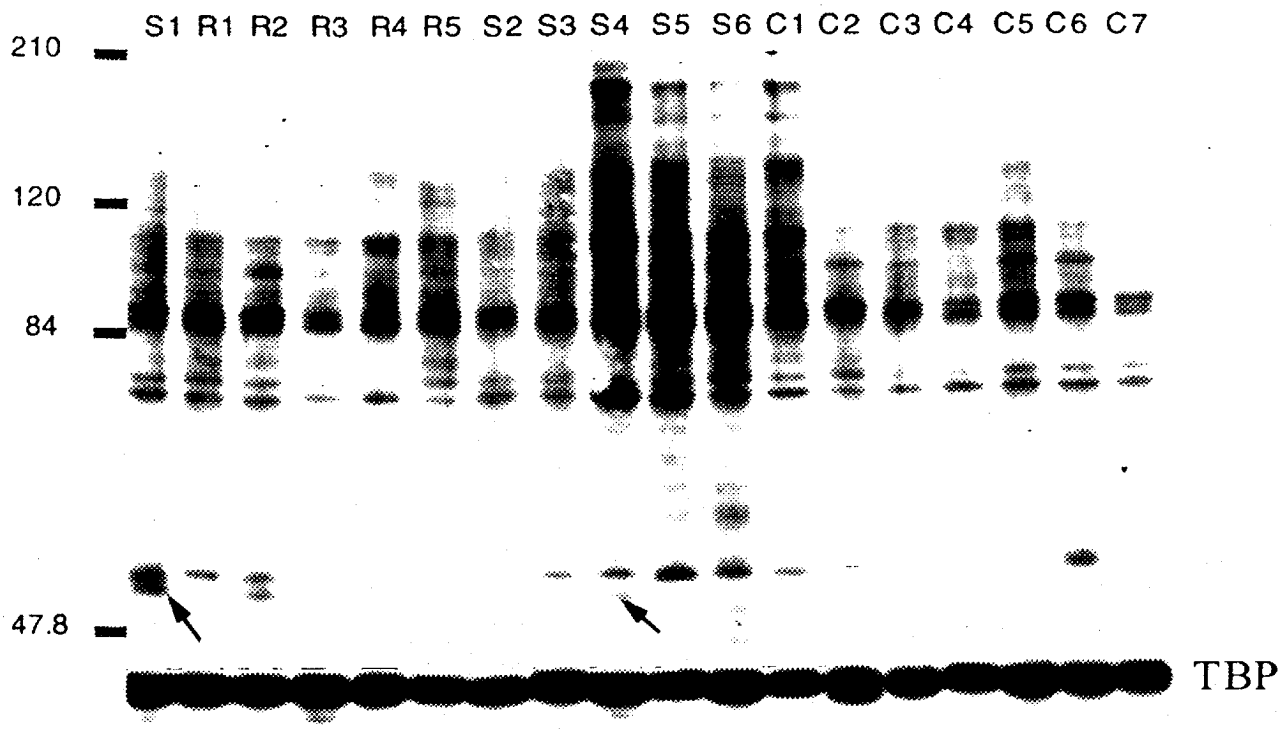

Figure 1 Western blot showing immunoreactivity in series of lymphoblastoid total protein extracts from lines established from schizophrenic patients (S), relatives (R) of one schizophrenic patient (S1) and healthy volunteers (C). All samples contained the same volume of protein extract. TBP, TATA-binding protein. The blot was exposed to the 1C2 antibody (50 ml, 1:5000) overnight at 4°C. Positions of the molecular size markers are indicated on the left of the figure. Arrowheads indicate the novel bands observed in the two unrelated schizophrenic patients (S1 and S4).

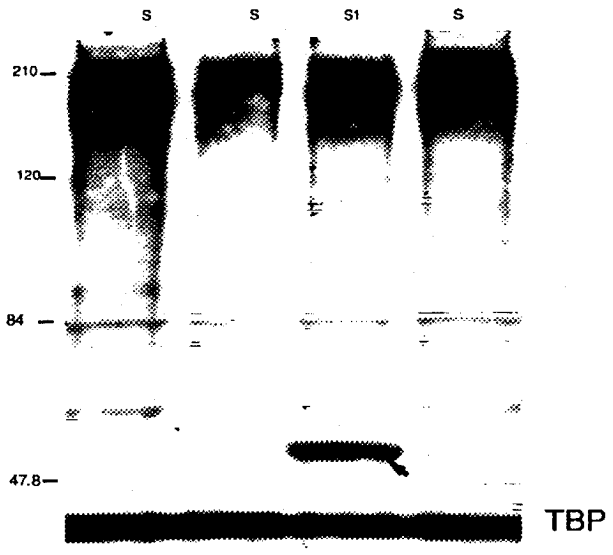

Figure 2 Western blot showing immunoreactivity in lymphoblastoid total protein extracts from lines established from four schizophrenic patients (S). All samples contained 50 micrograms of proteins. The blot was exposed to the 1C2 antibody (10 ml, 1:2000) for 1 h at room temperature. TBP, TATA-binding protein. Size marker is indicated on the left of the figure. Arrowhead indicates the novel band observed in S1.

two siblings (out of eight) who were diagnosed respectively with a major depressive episode and a postpartum depression. A novel slightly smaller band as that observed in the schizophrenic proband was detected in the sibling diagnosed with major depression (Figure 1, lane R2); no novel protein was detected in the sibling with postpartum depression. Three other non-affected siblings were tested and did not display any additional bands. No parents were available for testing (S1 parents were dead and S4 parents declined to participate).

Three other schizophrenic patients and one of the siblings of S1 (Figure 1, respectively lanes S2, S5, S6 and R2) showed weakly immunoreactive bands of smaller molecular weight (approximately 45–48 kDa). However, fainter immunoreactive bands in the same size range were observed in normal volunteers (Figure 1, lanes C2, C3, C6 and C7). The nature of these bands remains unclear.

Discussion

Two recent studies did not identify polyglutamine expansions in schizophrenic patients.[20,21] The first study by Schurhoff *et al*[20] included only three schizophrenic patients, none of whom had a family history of schizophrenia or related spectrum disorders. The second study by Jones *et al*[21] included 18 schizophrenic patients where repeat expansion detection

method showed CAG/CTG expanded genomic sequences. No expanded polyglutamine-containing proteins were detected. However, in contrast with our results, the only signal detected on the western blots was the TBP protein band, suggesting methodological differences with our study. Indeed, in our hands, several constant bands were observed. It is therefore possible that the results obtained by Jones *et al* were due to different experimental conditions where only the TBP, the primary epitope of the 1C2 antibody, could be detected (such as 30-s exposure to the autoradiographic film in Jones *et al* experiments compared to 1 min in our experiments). Using very stringent conditions will increase the specificity of the experiment allowing only detection of very expanded polyglutamine tracts (or the TBP). However, these same stringent conditions will result in a low sensitivity preventing the detection of weaker antigens which happens even in diseases with known CAG repeat expansions (Lopes-Cendes, unpublished data). Our results are consistent with recent findings by Neri *et al* (in press) who, using the 1C2 antibody detected an expanded polyglutamine-containing protein in two patients with childhood onset schizophrenia. The immunoreactive bands corresponding to this protein were in the same size range as the ones observed in our study.

Though the 1C2 antibody detects preferentially expanded polyglutamine tracts, smaller polyglutamine polymers are also detected by this antibody.[17] It is therefore difficult to be certain that the additional protein bands observed in the present study correspond to expanded polyglutamine tracts in the same size range as those causing neurodegenerative diseases. However, polyglutamine peptides with more than 35–40 polyglutamine repeats seem to be the preferential target for the 1C2 antibody (Lopes-Cendes *et al*, submitted). In addition, under some experimental conditions, the intensity of the abnormal band was similar to the immunoreactivity of the TBP, which is comparable to the intensity of abnormal bands observed in neurodegenerative diseases caused by CAG expansions (Figure 2). Together, these observations suggest that the additional protein bands observed in the two schizophrenic patients S1 and S4 and in one of their relatives diagnosed with depression, correspond to rare isoforms of a protein with either an enlarged (upper limit of the normal size range) or an expanded (CAG repeat > upper limit of the normal size range) polyglutamine tract.

Because there are slight differences in the size of the additional bands observed in S1, S4 and R2, we cannot exclude the possibility that the bands detected in the three subjects may represent different proteins. An alternative explanation to these size differences could be that they correspond to the same protein with different sizes of polyglutamine tract expansions (a short expansion will result in a smaller molecule and a weaker signal). If the latter hypothesis is true, the slightly smaller size of the novel band in R2 may reflect the fact that the schizophrenic proband inherited larger CAG repeat leading to a more severe phenotype than his sibling with major depression. In keeping with this hypothesis, S4, who showed a slightly smaller and weaker band than S1, did not show evidence for increased psychiatric morbidity in his relatives and had a later age-at-onset. Further experiments are required to establish whether these proteins are the same or not.

The co-occurrence of novel protein bands in two siblings suffering respectively from schizophrenia and major depression and its absence from three non-affected siblings suggests that the corresponding protein may contribute to the increased psychiatric morbidity in this family. This is in keeping with a study showing that expanded CAG repeats are not specific to schizophrenia but are also observed in patients with affective disorders.[11] This is also consistent with epidemiological data indicating that affective disorders and schizophrenia may share some genetic susceptibility factors.[22] The absence of the abnormal protein band in one sibling who suffered from postpartum depression may result from etiological heterogeneity, which is believed to be common in such prevalent conditions, or a smaller expansion not detected with the 1C2 antibody, a frequent occurrence even in the spinocerebellar ataxias.

Although we expected abnormal bands to be more frequent in severely affected and neuroleptic-nonresponsive patients, none of these tested positive for a novel band. It is difficult to speculate on the significance of this observation given the small size of the samples and the number of detected abnormal bands.

In conclusion, only two out of 57 unrelated schizophrenic patients showed an aberrant band detected by the 1C2 antibody. This observation indicates that the putative expanded polyglutamine-containing protein may contribute to the development of schizophrenia only in a small proportion of patients. This is consistent with the fact that schizophrenia is a complex disease where genes with a major effect are thought to be very rare.

Further investigation, particularly the identification and analysis of the gene coding for the protein corresponding to the abnormal bands and the screening of a larger group of schizophrenic patients and carefully matched controls, may help to clarify their role in schizophrenia and possibly affective disorders.

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